United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	Role of fuel composition on biomass smoke-induced pulmonary toxicity and allergic asthma in mice
LAPR Number:	19-08-002
Principal Investigator	Exemption 6
Author of this Document:	Exemption 6/RTP/USEPA/US
Date Originated:	08/02/2016
LAPR Expiration Date:	08/31/2019
Agenda Date:	08/10/2016
Date Approved:	08/17/2016
Date Closed:	

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6/RTP/USEPA/US by Exemption 6/RTP/USEPA/US	08/17/2016	DMR	
	Exemption 6 Exemption 6 Exemption 6/RTP/USEPA/US by Exemption 6/RTP/USEPA/US	08/17/2016	DMR	

Administrative Information

1. Project Title (no abbreviations, include species):
Role of fuel composition on biomass smoke-induced pulmonary toxicity and allergic asthma in mice

Is this a continuing study with a previously approved LAPR?
Yes

Please provide the previous LAPR# 18-08-002

2. Programatic Information

a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
ACE 183 (wildfire) PEP-1

b. What is the Quality Assurance Project Plan (QAPP) covering this project?
irp-nheerl/ephd/cib[redacted]/2015-001-01

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	**Exemption 6**	EPHD	MD
	Lotus Notes Address	***Branch***	
	[redacted]RTP/USEPA/US	CIB	

4. Alternate Contact:

Alternate Contact	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	**Exemption 6**	EPHD	MD
	Lotus Notes Address	***Branch***	
	[redacted]RTP/USEPA/US	CIB	

SECTION A - Description of Project

1. Explain the study objective(s) in non-technical language such that it is understandable by non-scientific

persons. Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research. If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.

LAPR 18-08-002 Exemption 6; approved September 2015) describes studies designed to assess the relative toxicity of biomass smoke emissions (i.e. from wildfires) from four distinct fuel types (pine, peat, chaparral and mixed conifer) using several cardiopulmonary, genotoxic, and neurobehavioral endpoints in Big Blue transgenic mice. Big Blue mice are used to assess mutagenicity (capacity to cause mutations or genetic alterations) and genotoxicity (mutations which cause retained alterations in DNA) of exogenous compounds on chromosomal DNA in the cells of any organ. LAPR 18-08-002 outlined inhalation exposures of Big Blue mice to the 4 types of biomass smoke emissions for 1, 4, and 13 weeks, with endpoints assessed 2 hours, 24 hours, or 7 days after exposure. Since LAPR 18-08-002 uses expensive and difficult to obtain transgenic mice which are exposed for many weeks, the present LAPR was designed to provide information on acute relative toxicity of biomass smoke emissions in normal mice and in a mouse model of allergic asthma. This information, besides being significant for assessing the immediate effects of wildfires on allergic asthma, will provide greater clarity with respect to appropriate exposure conditions for the subchronic inhalation protocols in LAPR 18-08-002.

Wildfires annually destroy thousands of homes, burn millions of acres, and threaten human life and health in the United States. Unfortunately, the scale and frequency of wildfires have been increasing over the past 30 years. Furthermore, since wood smoke is recognized by the World Health Organization (WHO) as a probable human lung carcinogen, health risks of short- and long-term exposures to wildfire smoke are becoming of growing concern not only for firefighters but also for the population impacted by smoke. Asthmatics are particularly sensitive to multiple types of air pollutants, including particulate matter (PM) and air toxic compounds, which are released in abundance by wildfires. The purpose of this project is to assess the relative acute toxicity of emissions from five distinct fuel types (pine, pine needles, peat, chaparral (eucalyptus), and red oak) and provide a ranking of effects on pulmonary and allergic asthmatic responses in Balb/cJ mice, a strain which is widely used for the assessment of allergic responses. Following further consultation with our collaborators at the Joint Fire Science Program Committee, a committee of professionals who study wildfire management, we added pine needles and red oak and deleted mixed conifer from the fuel types mentioned in LAPR 18-08-002 (that LAPR will be amended as necessary to reflect the updated fuel types as needed).

The rationale for this project is that biomass smoke emissions from different fuel types cause differential toxicity and could be used to identify toxic components within combustion emissions. Asthmatics may be particularly sensitive to the effects of biomass wildfire emissions. We will examine the toxicity of biomass smoke emissions in healthy and house dust mite (HDM)-allergic Balb/cJ mice, as a model of human allergic asthma. These studies will assess pulmonary function, biochemical, and cellular changes in the lungs of Balb/cJ mice after acute exposure to the biomass smoke to determine whether these biomass emissions affect lung respiratory function, and examine pathological mechanisms of inflammatory responses contributing to respiratory function changes.

Mice will be exposed to biomass smoke or air for up to 1 hour per day for a total of 2 days. Biomass smoke emissions will be generated from a glass tube furnace system in Exemption 6 Exemption 6, diluted and transported into the animal facility Exemption 6 Exemption 6 through a sealed penetration in the fume hood and directed to inhalation exposure chambers. Wildfire smoke emissions vary significantly depending on burning conditions. At high temperature (flaming condition), relatively little PM and carbon monoxide (CO) is released, but high amounts of volatile organic compounds (VOCs) are emitted. At lower temperatures (smoldering condition), high amounts of PM and CO are released, but these emissions may have relatively lower contents of toxic VOCs. We will test biomass smoke emissions released under these 2 burning conditions. Approximately 1-2 hours after the last exposure, mice will be tested for pulmonary function before being euthanized, and blood, lungs, heart and other tissues removed for analysis using immunologic, hematologic and pathologic techniques.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

The adverse effects of exposure to biomass smoke emissions are influenced by the responses of multiple organ systems, including the pulmonary, cardiovascular, and neurological systems. Furthermore, the development and exacerbation of allergic asthma involves integration of multiple organ systems, including the immune system (circulating lymphocytes and antibodies which promote allergic inflammation) and the respiratory system (airway smooth muscle and mucus producing cells which can restrict airflow). The complex interconnected nature of intact

physiological systems cannot be replicated using simple in vitro cell culture or tissue culture systems. In summary animal models must be used to ascertain the critical factors involved in the development of allergic responses and the effects of PM exposure on these responses,

b. Justify the species requested:

The BALB/c mouse model of allergic airway disease has many immune and pathophysiological endpoints which resemble those of human allergic asthma, including eosinophil inflammation of the lung (a white blood cell type typically involved in allergic responses), lung edema, serum IgE antibodies, increased pro-allergic cytokines (especially IL-5), and airway hyperresponsiveness to methacholine (higher lung airway sensitivity to a drug which induces airway narrowing). Well-defined immune and molecular reagents as well as standard operating procedures are available for mice in these studies. We have used this model in several studies and found a consistent inflammatory and physiological allergic asthmatic phenotype, and consequently use of this model in the present LAPR will allow us to compare responses to biomass smoke emissions with historical data.

3. How was it determined that this study is not unnecessary duplication?

The project assessing inhalation toxicity of biomass smoke emissions from different fuel types which represent wildland fires in the U.S in an allergic mouse model does not duplicate any existing published studies as determined by a Pub-Med search conducted on 8/3/2016. Key word searches included: wildfire, biomass smoke, allergic asthma, mouse. As expected, many studies were found examining the effects of tobacco smoke on allergic asthma, but no studies were found specifically assessing the effects of wildfire smoke.

SECTION B - In Vivo Procedures

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

Objective: The objective of this study is to provide comparative toxicity of five distinct biomass combustion emissions in allergic asthmatic mice. Pine, pine needles, peat, chaparral (eucalyptus), and red oak will be tested to represent western and southeastern wildland fires in the U.S. Simulated wildland fire burns will be conducted using a tube furnace system which consists of a quartz glass tube and controlled-speed traveling ring furnace. The tube furnace system is located in Exemption 6 Exemption 6 and biomass emission from the tube furnace will be delivered to an inhalation exposure tower in Exemption 6 Exemption 6 in which animals are exposed using rat nose-only tubes (essentially a whole body system with no restraint).

Timeline: The HDM allergic asthma model used here is the same as that used currently in our laboratory (active LAPRs 17-04-002 and 19-06-002 Exemption 6). Female Balb/cJ mice (7-10 weeks old, 18-23 g) will be sensitized intranasally to HDM (0.7 microgram of the antigen dermatophagoides pteronyssinus (DerP1)) in 50 uL saline vehicle or saline vehicle only on days 0 and 7 of the protocol (saline vehicle is used for the non-allergic groups). On day 21, all mice (allergic and non-allergic) will be challenged intranasally with HDM. On days 22 and 23, mice are exposed for 1 hour to the biomass smoke emissions or air only. Approximately 1-2 hours after the final exposure on day 23, mice are assessed for pulmonary function and responsiveness to methacholine aerosol using the emka-Scireq MSX Flexivent system in Exemption 6 Exemption 6 (Category D, see B.7. below) immediately before euthanasia as described below..

Exposure to biomass smoke emissions: The same mass of fuels to be combusted will be held constant at 15 g in order to compare the potency of different biomasses (e.g., pine, pine needles, peat, eucalyptus, and red oak). Dilution air will then be introduced to produce the smoke atmosphere. Mice will not be exposed to concentrations greater than 800 mg/cubic meter of PM and 800 ppm of CO (which would occur only under the smoldering condition). These concentrations are lower than those generated in many studies of tobacco smoke emissions in mice (PubMed search of "cigarette smoke mice" yields >2000 studies, often in mice exposed to tobacco smoke with these concentrations or higher of PM and CO for 5 days/week for up to 24 weeks). Dilution air will be manipulated and then after testing, held constant to maintain exposure concentrations below these levels. Mice will also be exposed to smoldering biomass emissions diluted to the same PM mass concentration as flaming biomass emissions PM concentration. Blood carboxyhemoglobin concentration in mice will be determined by submandibular bleeding in all cohorts of mice (Category C procedure) immediately after the final exposures.

The array of conditions tested in this LAPR include the following:

5 Fuel types: Pine, pine needles, peat, chaparral (eucalyptus), and red oak
5 Exposure conditions: (1) Smoldering, (2) Flaming, and (3) Air control; following assessment of these 3 conditions, run another comparison of (4) Smoldering reduced to the same PM concentration as Flaming, and (5) an additional air control.
2 Allergic status: Nonallergic, HDM-allergic

These factors (5 fuel types x 5 exposure conditions x 2 allergic statues = 50 groups) determine the scope of test condiitions. Further chemical analyses of the biomass smoke emissions in conjunction with biological endpoints will allow determination of the key components responsible for exacerbation of allergic airways disease endpoints.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.
It is necessary to include 8 mice per group to reduce the chance of false positive error. Experience with biological data including inflammatory cells, biochemistry, serum immunoglobulins, and pulmonary function, shows that this number of mice is necessary to distinguish group differences. The total number of mice for this LAPR is then (5 Fuel types x 5 conditions x 2 allergic statues = 50) x 8 mice per group = 400 total mice.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:		
D) Potential pain/distress relieved by appropriate measures:	400	
E) Unrelieved pain/distress:		

4. Does this LAPR include any of the following:
☐ Restraint (>15 Minutes) ☐ Survival surgery
☐ Food and/or water restriction (>6 Hours) ☒ Non-survival surgery

a. Please provide a scientific justification. Describe how animals will be monitored, how health status will be tracked, and what records will be maintained.
The use of non-survival surgery is required for the Flexivent procedure (see Category D procedure in B.7.a. below), because this is the only method of obtaining accurate lung mechanical properties (such as airflow resistance and lung compliance or distensibility) in the mouse. Following urethane anesthesia (see B.7.a.), mice are carefully assessed to determine the proper level of anesthesia using a gentle toe pinch. If no movement is observed, mice undergo a tracheal cannulation procedure, placed on the Flexivent ventilator in a plethysmograph (body box) which is programmed to give proper tidal volume according to body weight, and assessed again for any signs of recovery from anesthesia. If limb or body movements are observed, the plethysmograph cylinder can be removed and either further anesthesia administered (urethane, 1000 - 2000 mg/kg), or the mouse will be removed from the system and euthanized by overdose of sodium pentobarbital (150 - 250 mg/kg, i.p.). Heart rate and body temperature for each mouse are determined before and after the Flexivent procedure and recorded in a logbook. Stability of heart rate and temperature following the Flexivent procedure supports evidence for a successful procedure with potential pain or distress relieved by the anesthesia.

5. Category C procedures. Describe each procedure separately, include details on the following:
a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):
HDM: Mice will be anesthetized with isoflurane vapor (2-4% vapor setting, 2 L/min in 100% oxygen, approximately 30 seconds to 2 minutes to induce anesthesia) for a total of 3 times during the experimental protocol in order to be exposed to HDM as follows. (1, 2) On Days 0 and 7, mice will be anesthetized and sensitized intranasally to the HDM (0.70 microgram antigen in 50 uL saline vehicle) or given saline vehicle only. (3) Two weeks later (day 21), all mice will be anesthetized and challenged with HDM (0.70 microgram antigen in

saline vehicle). For dosing, mice are observed until breathing becomes shallow and regular (~ 120 breaths/minute). Mice are removed from the anesthesia box and a sterile tip pipet is used to bring the solution close to the nares. Approximately half the volume is sniffed into each nostril.

Biomass smoke inhalation: Mice will be exposed to biomass emissions for up to 1 hour per day on two consecutive days (days 22 and 23 of protocol) using an inhalation exposure tower in which animals are exposed using rat nose-only tubes (essentially a whole body system with no restraint). Prior to inserting the mice into the chambers, their eyes will be coated with eye lubricant to avoid any ocular irritation caused by smoke exposure. Exposure concentrations will be up to 800 mg/cubic meter of PM and 800 ppm of CO under smoldering condition, and much lower concentrations under flaming condition. Air-exposed mice will be exposed to filtered air only in an identical system. Continuous gas and aerosol sampling for CO, carbon dioxide, oxygen, total hydrocarbon, and particle mass concentration will be conducted from the chamber. The port airflow rate will be maintained between 500 - 1000 mL/min which is well above the respiratory rate for any individual animal being exposed, and ensures a constant atmospheric concentration of pollutants. The biomass smoke concentration entering the chamber will be precisely controlled and monitored, and the flow maintains a slightly positive static pressure in the chamber. To ensure no fugitive emissions and a safe environment for the exposure operators a secondary containment enclosure will be used. In both cases the air temperature will be controlled in a range of 70-75 F, and humidity will be maintained between 30-70%. Mice will be observed continuously during exposure periods.

b. Survival Blood Collections (method, volume, frequency):

Each mouse (20-25 g) will be bled for one time from the facial vein in order to measure carboxyhemoglobin immediately after the final day of exposure. Mice will be removed from the exposure chamber and gently restrained. The whorl (or cowlick) will be identified on the mouse cheek and the puncture performed using a 3 mm sterile lancet. Blood (up to 150 microliters) will be collected in a pipette and transferred to a container for assay. Pressure will then be applied with sterile gauze until bleeding has stopped.

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):

Pulmonary function tests: Airway responsiveness in unanesthetized, unrestrained mice. Immediately before and after the first exposure (Day 1 only), assessments will be obtained on all mice using a whole body plethysmograph (emka-Buxco) system to measure ventilatory parameters and the enhanced pause (Penh), an index of airflow limitation and a surrogate for bronchoconstriction. This system allows complete freedom of movement in a small chamber (2" high x 3.5" diameter). Additional measured parameters include tidal volume, frequency, inspiratory and expiratory flow rates, inspiratory and expiratory times, and minute ventilation. Accumulation of individual ventilator function data at these 2 time points will be collected for 30 min following a 5 min acclimation period for each mouse in its individual. The whole body plethysmography will take place in A-building. The testing will not use any aerosol challenges (e.g. methacholine) and will be conducted by Exemption 6Exemption 6Exemption 6

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:

Using the inhalation exposure tower system, mice will be placed in rat tubes (10" length, 3" diameter) where they are free to turn around unimpeded. Duration of exposure is up to one hour per day for 2 days.

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):

f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:

Animals of the same group will be housed together, and so identified by cage card. Individual mice within the group will be identified by tail marks (1 to 4). Animals will be placed in exposure tubes with marked tape to account for individual numbers. These procedures will be sufficient to identify mice in these short term exposures. Animals will be monitored at key points of the protocol (sensitization, during inhalation exposure, and pulmonary function testing) for any signs of ill health by Exemption 6Exemption 6 to ensure they fully recover before being returned to animal housing.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):

c. Testing methods:

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

All mice in this LAPR will be tested by the emka-Scireq MSX Flexivent system. Non-survival Flexivent pulmonary function testing provides objective measures of pulmonary pressure and dynamic compliance which leads to calculation of airway resistance. Airway responsiveness to methacholine (MCh) aerosol will be assessed in 4 mice at once on a multiple platform system. Mice will anesthetized with urethane (1 - 2 g/kg intraperitoneal, about 0.3 ml for a 20 gm mouse) and carefully tested to determine lack of responses to toe pinch. (Urethane is known to preserve cardiovascular function). This procedure takes approximately 50 minutes total to complete for the 4 mice, from initial anesthesia to the recording of the response following the last exposure to methacholine aerosol.

The instruments and surrounding area will be clean and the surgeon Exemption 6 will wear gloves. The tracheotomy procedure is as follows: Anesthetized mice are placed on thermal support (circulating water bath pad or equivalent), the fur is clipped in the neck area with a small electric razor, and then 70% alcohol is applied to the area. Small surgical scissors are used to cut the skin layer in one clean line parallel to the trachea, and then to gently separate the submandibular and parotid glands, also parallel to the trachea, to expose the muscle layer overlying the trachea. If voluntary movement is noted during the incisions, the procedure is halted until a deeper plane of anesthesia is achieved, and if necessary another 0.1 ml of urethane anesthetic will be administered i.p. The scissors are then used to separate the muscle layer parallel and directly over the trachea, thus revealing the trachea. Extensive experience with this technique allows the trachea to be revealed without any bleeding. Microscissors are then used to cut half-way through the trachea between the 4th to 5th ring from the larynx. A 20 g blunt tip luer cannula is inserted into the trachea, and then tied into place with 3-0 braided non-absorbable silk surgical suture.

With the Flexivent MSX, anesthetized cannulated mice will be attached to the ventilator port, the cylinder is attached to the faceplate, the mouse is further monitored for required anesthesia, and breathing volume and pressure are monitored on the computer screen. Mice are ventilated with 100% oxygen at 150 breaths per minute. If limb or body movements are observed, the plethysmograph cylinder can be removed and either further anesthesia administered (urethane, 1000-2000 mg/kg), or the mouse will be removed from the system and euthanized by overdose of sodium pentobarbital (150 - 250 mg/kg, i.p.). After ensuring proper anesthesia, neuromuscular blockade is administered i.p. (pancuronium bromide, 0.8 mg/kg i.p., about 0.2 ml for a 20 gm mouse) to eliminate skeletal muscle movement which interferes with airway measurements. ECG leads will then be attached to 3 limbs in a lead II configuration for 30 seconds to determine heart rate

(beats/second). A rectal probe is inserted to record body temperature. Following recording of heart rate and temperature, the leads and probe will be removed and the cylinder closed. When all 4 mice are surgically prepared and breathing on the MSX ventilator, and baseline temperature and heart rates have been recorded, mice are ready for methacholine aerosol challenge. Up to 4 doses (ranging from 5 - 40 mg/ml) of methacholine are aerosolized to assess lung functional changes. At the end of the assessment (about 30 minutes), each cylinder is removed one at a time, and ECG leads attached again for 30 seconds to determine heart rate for each mouse. The heart rates before and after the methacholine challenge assessment will be recorded and used to ensure efficacy of urethane as adequate anesthesia. Our experience under LAPR 17-04-002 and 19-06-002 is that the MSX procedure works well with very stable heart rate after the methacholine challenge.

Animals will be monitored during the procedure and should they show any signs of recovery from anesthesia, (e.g. eye and body movement) then the animal will be euthanized by overdose of sodium pentobarbital (150 - 250 mg/kg, i.p.). At completion of experiments the mice will be humanely euthanized while under urethane anesthesia by exsanguination to recover blood, and vital organ section (cutting of descending aorta and kidney). Bronchoalveolar lavage fluid, nasal lavage fluid, and lung and nasal tissue will then be collected for cytological, biochemical, and histopathological assessments.

Records of the use of pancuronium bromide and urethane are kept for each animal and are available for inspection.

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

Mice are given urethane (1-2 g/kg; 0.3 ml for a 20 gm mouse) by the intraperitoneal route once prior to surgery to achieve a proper level of anesthesia.

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

Not applicable.

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):

Not applicable.

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?

○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:

Not applicable.

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

Biomass smoke may cause airway irritation and lung inflammation. Acute effects are expected to recover after the exposure although it is not known whether repeated exposures will result in cumulative injury. As noted previously, hundreds of tobacco smoke studies have been conducted with the target levels of PM and CO, and mice typically become tolerant to these exposures. During smoke exposures, mice will be individually observed through the exposure chamber window and any evidence of ill health such as labored breathing will be noted. In such cases, animals will be removed from the study and/or euthanized as per advice of the staff veterinarian.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

Animals will be monitored each day during and after exposure and any animals displaying signs of illness (huddling, isolation with ruffled exterior, shivering, development of hindered movement, etc) will be assessed in consultation with on-site veterinarian. Development of abdominal mass, ulceration, or untreatable malocclusion are common criteria for removal of the animal from the study

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the

sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

PubMed (covering 1950 to the present) was searched 8/2/2016 for alternatives to pain and distress for this procedure, using the following key words in combination with mouse pulmonary function: Animal testing alternative, In Vitro (method, model, technique), Welfare, pain, stress, distress. No suitable alternatives to the flexivent procedure were identified..

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study: 400

b. Animals to be transferred from another LAPR:

LAPR Number that is the source of this transfer:

c. Animals to be transferred from another source:

d. Offspring produced onsite (used for data collection and/or weaned):

e. TOTAL NUMBER of animals for duration of the LAPR 400

2. Species (limited to one per LAPR): Mouse/Mice

3. Strain: BALB/C mouse/mice

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

Not applicable

4. Sources of animals:

Jackson Laboratories, ME or CA sites

5. Provide room numbers where various procedures will be performed on animals:

Building A 5th floor housing suite to be determined, biosafety cabinet - HDM sensitization. Exemption 6 Exemption 6: Whole body biomass smoke exposures, Exemption 6 Exemption 6 flexiVent pulmonary mechanics and necropsy.

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

No ***Room Numbers:***

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

Animals will be transported within building A, or from building A to building B in transfer cages with filter tops.

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

No unusual housing requirements or acclimation are needed.

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program

Office (ARPO)

No special assistance of animal contract staff is required other than the usual animal care.

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

Animals will be group-housed (4 per cage or fewer) in solid bottomed cages with beta chip bedding or other approved bedding. All mice will have access to enviro-dry with nestlets for enrichment purposes.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

Isoflurane (maximum dosing has not been quantified, but is dependent on breathing rate and partition coefficients of gas to blood and blood to brain) - The LD50 of isoflurane is 16800 ppm for over 3 hours, whereas we are exposing mice to 1 - 3 % isoflurane (20000 ppm) for less than 2 minutes.

Physiological saline, pharmaceutical grade: used for HDM and methacholine solutions.

House dust mite (HDM) (maximum dosing = 0.7 ug of the antigen Dermatophagoides pteronyssinus, or 50 ug total protein per dose; no LD50 is available). No HSRP is required, however, since handling HDM could cause allergic sensitization, the user will resuspend the material in sterile saline in a biological safety cabinet under sterile conditions. Handling the material in saline suspension will prevent exposure to the user. Unused material (14 ug HDM/ml) will be disposed of with laboratory waste. Prior experience has shown that these doses are well-tolerated in mice.

Pine and pine needles were provided by the Fire Science Laboratory in Missoula, Montana, and peat, eucalyptus, and red oak were obtained by EPA locally or ordered from other sources. Biomass combustion exposures will be up to 800 mg per cubic meter for total particulate (inhalation, 1 hour/day). There is no LC50 for biomass smoke inhalation exposures although studies have been reported with minor adverse effects for cigarette smoke at 800 mg per cubic meter. The LC50 for CO in mice is 2444 ppm/4 hours. CO concentrations will be maintained below 800 ppm to avoid acute toxicity during exposures and no mortality is expected. Chamber CO will be monitored continuously to prevent acute toxicity, and blood carboxyhemoglobin will be measured immediately after final exposure as a biomarker of exposure. Inhalation exposures will be conducted in inhalation chamber either operated under negative pressure or in a secondary enclosure to maintain a negative pressure for the chamber. For the inhalation system, a 5 minute flush period after exposure will be observed and inhalation chambers will not be opened until particle concentrations which are monitored continuously return to ambient levels. Gloves, lab coat and mask will be worn by personnel during all handling, transportation and experimental procedures including post-exposure cleanup.

Urethane (1000 - 2000 mg/kg; mouse oral LD50 = 2500 mg/kg; IARC group 2A carcinogen) is used for anesthetizing mice tested on the flexivent pulmonary function testing apparatus (section A above). In the same procedure, pancuronium bromide (0.8 mg/kg) is used to eliminate skeletal muscle movement. The approved HSRP for these 2 agents is #687 (Allergenicity of Platinum Salts). The i.p. LD50 for urethane in mice is not available.

Pancuronium bromide (pharmaceutical grade, maximum dosing = 1 mg/kg; LD50 = 128 ug/kg mouse i.p. due to respiratory skeletal muscle paralysis).

Methacholine: (pharmaceutical grade; rat oral LD50 = 750 mg/kg). The mice will be challenged with increasing doses of methacholine via aerosol exposure (max) concentration 50 mg/ml in saline x 1 minute exposure). Prior experience has shown that these doses are well-tolerated in mice. Gloves, lab coat and mask will be worn by personnel during all experimental procedures.

Pentobarbital and phenytoin mixture. Pentobarbital maximum dosing 150 - 250 mg/kg; mouse oral LD50 = 137 mg/kg. Phenytoin maximum dosing >20 mg/kg; mouse oral LD50 = 150 mg/kg. The material will be kept in a locked drawer, and an inventory maintained. This is only used when euthanasia is required for a suffering mouse (use not anticipated).

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.

All compounds above will be pharmaceutical grade, except house dust mite (a biological agent not available as pharmaceutical grade), biomass smoke emissions (environmental pollution samples), and urethane (unavailable as pharmaceutical grade).

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

Not applicable

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

Normal personal protective equipment (PPE) precautions will be observed throughout (gloves mask, labcoat, safety glasses). Inhalation exposures will be conducted under negative pressure to ensure safety of personnel.

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Project management, blood collection, pulmonary function assessment, necropsy	Over 20 years experience in inhalation toxicology. Completed all NHEERL-required training.

Exemption 6	Post-Doc	Input into study design, lung function, blood collection, animal handling and necropsy	Over 7 years experience in pulmonary and cardiovascular experimentation using mice and rats. Has taken all required NHEERL training.
Exemption 6	Post-Doc	Input into study design, exposure methodology, blood collection, animal handling and necropsy	7 years animals experience. Has extensive expertise on specific techniques Completed all required NHEERL training.
Exemption 6	Associate Principal Investigator	Assist with study design, animal handling and necropsy.	Completed all NHEERL-required training; has over 20 years of experience conducting animal inhalation toxicology
Exemption 6	Associate Principal Investigator	Pulmonary toxicity assessment and veterinary input	Over 20 years experience in animal toxicology. Completed all NHEERL-required training.
Exemption 6	Technical Staff	Toxicity assessment, bleeding and necropsy	Over 20 years experience in inhalation toxicology. Completed all NHEERL-required training.
Exemption 6	Technical Staff	Assistance with exposure methodology	Over 20 years experience in aerosol and inhalation testing. Completed all NHEERL-required training.
Exemption 6	Technical Staff	Assistance with exposure methodology	Over 20 years experience in animal toxicology. Completed all NHEERL-required training.
Exemption 6	Technical Staff	Assistance with exposure and physiology testing	Over 20 years experience in inhalation toxicology. Completed all NHEERL-required training.
Exemption 6	Technical Staff	Toxicity assessment, bleeding and necropsy	Over 20 years experience in animal toxicology. Completed all NHEERL-required training.
Environmental Public Health Division	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year

2. Breeding protocols and recordkeeping

3. Methods for monitoring genetic stability

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Mice will be euthanized immediately after the testing for lung mechanics on the Flexivent (day 23

of the protocol).

2. Describe the euthanasia techniques:

Method(s): Anesthesia plus vital organ transsection
Agent(s): Urethane, or sodium pentobarbital/phenytoin sodium mixture
Dose (mg/kg): 1000-2000 mg/kg (urethane), 150 - 250 mg/kg (pentobarbital)
Volume: (20 gm mouse) 0.32 ml (urethane), 0.1 ml (pentobarbital)
Route: intraperitoneal

Source(s) of information used to select the above agents/methods:

_ Veterinary Staff, 2013 AVMA Guidelines on Euthanasia.

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).

4. Describe how death is to be confirmed.

Vital organ section

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.

Euthanized by Animal Care Contractor

The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?

● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	08/03/2016

Submitted: 08/03/2016

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	***Approval Date***	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	08/03/2016 by Exemption 6 Exemption 6 RTP/USEPA/US	Exemption 6 ***Lotus Notes Address*** Exemption 6 Exemption 6 /RTP/USEPA/US	***Branch***	MD ***Submitted to Branch Chief for Approval*** 08/03/2016 03:11 PM

ATTACHMENTS

19-08-002 PI resp.pdf

Actions

First Update notification sent: 07/03/2017
Second Update notification sent:
First 2nd Annual notification sent:
06/28/2018
Second 2nd Annual notification sent:

1st Expiration notification sent:
2nd Expiration notification sent:

History Log: